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Synthesis and Binding Activity of Endomorphin-1 Analogues Containing β-Amino Acids

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Abstract—Endomorphin-1 (Tyr-Pro-Trp-PheNH_2) has been proposed as the most potent endogenous ligand of the μ-opioid receptors. In this paper, we describe the synthesis of some endomorphin-1 based tetrapeptides in which a residue of the sequence Tyr-Pro-Trp-PheNH_2 is replaced by the corresponding β-isomer. These novel peptides showed different affinities for the opioid receptors labeled with [^{3}H]-DAMGO in rat brain membranes, depending on the β-amino acid. In particular, the tetrapeptide containing β-Pro (Tyr-β-(*R*)-Pro-Trp-PheNH_2) displayed a higher affinity than endogenous endomorphin-1, as revealed by their K_i values (0.33 and 11.1 nM, respectively).

Introduction

Morphine is the pharmacologically active component of opium, a poppy plant extract which has been used for centuries as a drug, and is the most widely-used remedy for pain relief. The analgesic efficacy is accompanied by undesirable side effects such as physical dependence, tolerance, reward-seeking behaviour, breath depression, etc.[1] All these effects are mediated by a family of receptors coupled to G-proteins, the μ-, δ-, and κ-opioid receptors.[2] Many of the pharmacologically useful opioids, including morphine, are mainly selective towards the μ-receptors.[3] Some endogenous morphine-like substances were discovered in the mammalian brain in the 1980s, namely the δ-selective enkephalins and the κ-selective dynorphins,[4] while the μ-selective endomorphin-1 (Tyr-Pro-Trp-PheNH_2) and -2 (Tyr-Pro-Phe-PheNH_2) were discovered by Zadina et al. in 1997.[5] For their potency, efficacy, and selectivity, these latter are proposed as the endogenous μ-opioid receptor ligands. Recently, it has been demonstrated that the residues contained in the amino-terminal domain of μ-receptors are of particular importance in the composition of the binding pocket for endomorphins and other agonists.[6] An interesting study of endomorphin-1 conformation in comparison to other μ- or δ-selective peptide ligands, has confirmed the hypothesis of the existence of different selectivity pockets among the opioid receptors.[7]

It has been also proposed that endomorphin-1 and -2 may act through two distinct μ_1- and μ_2-opioid receptor subtypes, respectively.[8] Similarly, also for a morphine metabolite, morphine-6-β-D-glucuronide,[9] it has been proposed that it may act through distinct μ-receptor subtypes.[10]

A major challenge in opioid peptide chemistry and pharmacology is the possibility to develop novel analgesics mimicking the endogenous opioid ligands instead of morphine. However, since these peptides are degraded by enzymes, their biological activity is of little therapeutic interest. For this reason, it is often convenient to consider the corresponding peptidomimetics or more stable peptide analogues, by modifying the original structure of endogenous peptides. In fact, numerous analogues to endogenous enkephalins and dynorphins have been synthesized and investigated.[11]

Moreover, experimental and theoretical studies of affinity variations related to structure mutations are of great help to understand the structural basis for ligand recognition and selectivity of opioid receptors, and structure–function relationships of the ligands. For instance, μ-opioid receptor selectivities and affinity strongly depend on the presence of: (1) Tyr at the N-terminus position; (2) Pro or D-Ala in the second position; (3) a lypophilic residue as third and fourth amino acid. Moreover, amidation at the C-terminus seems to

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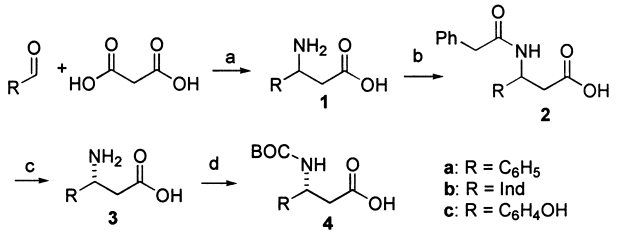
be important to protect against proteolytic degradation. Endomorphin-1 and -2 result among the most potent of the endogenous tetrapeptidic ligands tested.[5a,12]

In an effort to prepare endomorphin-1 analogues, we directed our work to the synthesis of tetrapeptides containing β-amino acids. In several cases, the substitution of an α-amino acid for their β-isomer in biologically active peptides resulted in an increased activity and enzymatic stability.[11b,c] β-Peptides, formed by β-amino acids having an extra backbone carbon, have been intensely studied for years to discover stable well-ordered secondary structures.[13] On the contrary, the use of isomeric β-amino acids to induce punctual mutations in peptides is much less described. In this study, we describe the preparation of some tetrapeptides in which a residue of the series Tyr-Pro-Trp-PheNH_2 is replaced by the corresponding β-isomer. In this way the new tetrapeptides maintain the size of the parent endomorphin. Finally, we present the affinities to μ-receptors through binding assays performed on rat brain membranes.

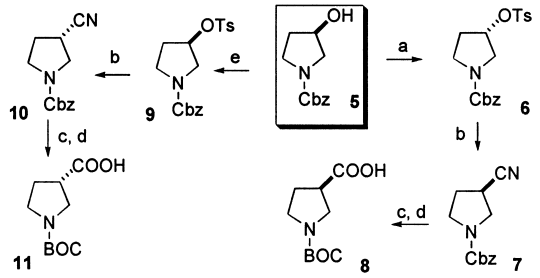
Chemistry

For the synthesis of isomeric β-amino acids we used modified versions of procedures reported in the literature.[14] Isomeric optically pure (*S*)-*N*-*tert*-butyloxycarbonyl β-Phe **4a**, β-Trp **4b**, and β-Tyr **4c** were prepared by enzymatic resolution. The racemic β-amino acids **1** were prepared[14c] by refluxing the appropriate aldehyde, malonic acid and ammonium tetraacetate in ethanol. The racemates were treated with phenylacetyl chloride in the presence of triethylamine, giving *N*-phenylacetyl amino acids **2**. The optically pure β-amino acids **3** were obtained by hydrolysis with penicillin G-acylase (PGA) immobilized on Eupergit.[15] Final amino group protection to *N*-BOC β-amino acids **4** was performed with di-*tert*-butyl dicarbonate (Scheme 1).

Isomeric optically pure *N*-*tert*-butyloxycarbonyl β-Pro was prepared both in (*S*) and (*R*) configuration starting from the same, commercially available (*R*)-3-hydroxy pyrrolidine. To obtain (*R*)-β-proline, *N*-benzyloxycarbonyl (*R*)-3-hydroxy pyrrolidine **5**[16] was treated with zinc tosylate under Mitsunobu conditions,[17] and this first inversion of configuration gave (*S*)-tosyl derivative **6** (Scheme 2). A second inversion of configuration with KCN[16] gave (*R*)-3-cyano pyrrolidine **7** ($[\alpha]_D^{25} = -22.0$, *c* 0.7, $CHCl_3$). After HCl hydrolysis, protection with



Scheme 1. Reagents: (a) CH_3COONH_4, EtOH, reflux, 6 h, 50–75%; (b) $PhCH_2COCl$, Et_3N, H_2O/acetone, 0 °C, 2 h, 75%; (c) 10% PGA, pH 7 buffer:MeOH 10:1, rt, 4–8 h, 40–50%; (d) $(BOC)_2O$, Na_2CO_3, H_2O/acetone, 0 °C, 2 h, 95%.



Scheme 2. Reagents: (a) Ph_3P, $Zn(OTs)_2$, DEAD, benzene, rt, inert atmosphere, 2 h, 60%; (b) KCN, DMSO, 80 °C, 4 h, 70%; (c) 6 N HCl, reflux, 6 h, 95%; (d) $(BOC)_2O$, Na_2CO_3, H_2O/acetone, 0 °C, 2 h, 95%; (e) TsCl, Et_3N, DMAP, CH_2Cl_2, 0 °C–rt, 3 h, 90%.

di-*tert*-butyl dicarbonate afforded (*R*)-*N*-*tert*-butyloxycarbonyl β-proline **8** ($[\alpha]_D^{25} = -8.0$, *c* 0.5, $CHCl_3$).

To prepare (*S*)-β-proline, the alcohol **5** was tosylated as usual to afford **9** which was treated with KCN,[16] giving the (*S*)-3-cyano derivative **10** with inversion of the configuration ($[\alpha]_D^{25} = +25.4$, *c* 1.3, $CHCl_3$).

Treatment with HCl and final protection with di-*tert*-butyl dicarbonate gave the (*S*)-*N*-*tert*-butyloxycarbonyl β-proline **11** ($[\alpha]_D^{25} = +7.7$, *c* 0.8, $CHCl_3$) (Scheme 2).

All tetrapeptides were prepared in solution using a convergent approach. As a general procedure,[18] the peptide coupling was performed by stirring overnight the HCl salt of the amino amide, the *N*-*tert*-butyloxycarbonyl amino acid, triethylamine, 1-hydroxy-1*H*-benzotriazole, the HCl salt of 1-[3-(dimethylamino) propyl]-3-ethylcarbodiimide, in a 9/1 mixture of CH_2Cl_2 and DMF at 0 °C.

Peptides were obtained pure by flash chromatography over silica gel with yields from 60 to 90%.

N-*tert*-Butyloxycarbonyl group deprotection was performed by treatment with sat. HCl in dioxane[18] and the resulting HCl peptide salts were used without purification for the next coupling. Finally, HCl tetrapeptide salts were purified by recrystallization. Purities were determined by analytical HPLC on an RP ODS Hypersil column by two solvent systems: A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile, with gradient 95% A to 80% B in 50 min at a 2.0 mL/min flow. All new compounds were characterized by IR and ^{1}H NMR; HCl tetrapeptide salts were characterized by ^{1}H NMR, and FAB-MS. In this way, we prepared the tetrapeptides **14**–**19** reported in Table 1, and we tested their affinities towards μ-receptors as reported in the next session.

Results and Discussion

Membrane preparations and determination of protein content

Rat brain, without cerebellum, was weighed and homogenized in 0.32 M sucrose at 4 °C. The homogenate was centrifuged (2000 rpm, 10 min, 4 °C), and the surnatant was in turn centrifuged (19000 rpm, 20 min, 4 °C). The

resulting pellet was suspended in 10 vol TRIS (pH 7.5) 50 nM/NaCl (binding buffer) and incubated for 1 h at room temperature to remove any endogenous opioid ligands. After a final centrifugation (19000 rpm, 20 min, at 4 °C), the pellet was stored at −80 °C for up to 1 week.

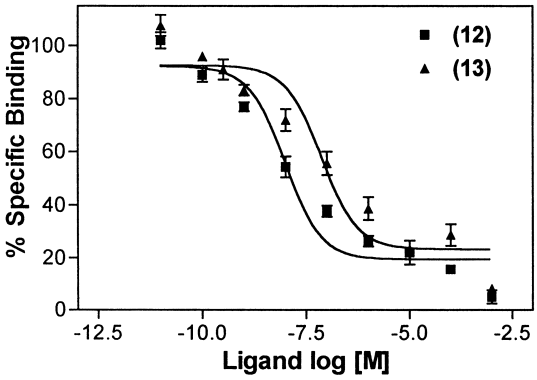


Figure 1. Competition curves of DAMGO (**12**) and endomorphin-1 (**13**) with [^{3}H]-DAMGO for its specific binding sites in rat brain membranes. Membranes were incubated with various concentrations of ligands (10^{-12}–5×10^{-2}) and 2 nM DAMGO for 60 min at rt. In some cases, S.E.s were smaller than the symbols.

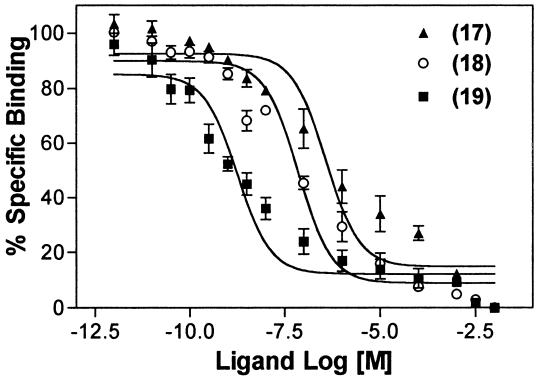


Figure 2. Competition curves of Tyr-D-Pro-Trp-PheNH$_2$ (**17**), Tyr-β-(*S*)-Pro-Trp-PheNH$_2$ (**18**), Tyr-β-(*R*)-Pro-Trp-PheNH$_2$ (**19**), with [^{3}H]-DAMGO for its specific binding sites in rat brain membranes. Membranes were incubated with various concentrations of ligands (10^{-12}–5×10^{-2}) and 2 nM DAMGO for 60 min at rt. In some cases, S.E.s were smaller than the symbols.

Protein concentration was determined according to Lowry et al.[19] [^{3}H]-DAMGO was used as a μ-specific radioligand (2 nM, specific activity 54 Ci/mmol, K_d = 0.38 nM, B_{max} = 48 fmol/mg protein), and non-specific binding was determined with 10 μM DAMGO. Incubation buffer consisted of 50 nM Tris (pH 7.5, 25 °C). To prevent protein degradation, protease inhibitors were added to binding buffer (captopril, 25 μg/mL; bacitracin, 80 ng/mL; and leupeptine, 10 μg/mL; bestatin 10 μg/mL). δ- and κ-Opioid receptors were saturated with 0.01 M DADLE and 0.01 M U50, 488, respectively.

The mixture (1 mL) was incubated for 1 h at room temperature, than it was filtered under vacuum through a glass fiber and washed with cold binding buffer. The ligand–receptor complex radioactivity retained in the filter was measured by liquid scintillation spectrometry using a Beckman scintillator after 12 h incubation in scintillation cocktail. All assays were performed in triplicate, and repeated at least three times.

The K_i values measured for DAMGO (**12**)[11a] and for endomorphin-1 (**13**)[5c–e] were in agreement with the literature (Fig. 1 and Table 1). The results in Table 1 show that changing Phe, Trp, or Tyr with β-Phe (**14**), β-Trp (**15**), or β-Tyr (**16**), respectively, caused a significant decrease of affinity in comparison to endomorphin-1 (**13**). In contrast, substitution of Pro with β-(*S*)-Pro gave a tetrapeptide (**18**) which retained almost the same affinity as endomorphin-1 (**13**), while substitution of Pro with β-(*R*)-Pro gave a tetrapeptide (**19**) which was 34-fold more potent than the endogenous ligand (**13**) (Fig. 2 and Table 1). It should be noted that β-(*R*)-Pro and not β-(*S*)-Pro maintains the same spatial disposition as natural Pro. The marked affinity decrease measured for the tetrapeptide containing α-D-Pro (**17**) relative to endomorphin-1 (**13**) confirmed that the inversion of the configuration in Pro produces an affinity loss,[20] as observed for **18** and **19**.

Conclusion

Our results suggest that the substitution of Phe, Trp, or Tyr residues with a β-analogue in endomorphin-1 sequence could disallow specific binding interactions.

Table 1. Affinities and Hill slopes of ligands **12**–**19** for [^{3}H]-DAMGO binding sites in rat brain membranes. Means ± S.E. of three experiments performed in triplicate

Compound	K_i (nM)[a]	IC_{50} (nM)[a]	n_H[a]
DAMGO (**12**)	1.64 (±0.33)	9.89 (±0.67)	0.88 (±0.10)
Endomorphin-1, Tyr-Pro-Trp-PheNH$_2$[b] (**13**)	11.1 (±4.8)	56 (±20)	0.78 (±0.08)
Tyr-Pro-Trp-βPheNH$_2$[c] (**14**)	908 (±50)	680 (±50)	nd
Tyr-Pro-βTrp-PheNH$_2$[c] (**15**)	$>10^3$	nd	nd
βTyr-Pro-Trp-PheNH$_2$[c] (16)	$>10^3$	nd	nd
Tyr-D-Pro-Trp-PheNH$_2$[c] (**17**)	54 (±19)	470 (±170)	0.66 (±0.06)
Tyr-β-(*S*)-Pro-Trp-PheNH$_2$[c] (**18**)	10.4 (±1.5)	72.0 (±11.0)	0.81 (±0.05)
Tyr-β-(*R*)-Pro-Trp-PheNH$_2$[c] (**19**)	0.33 (±0.10)	1.80 (±0.15)	0.77 (±0.09)

[a]Values are the mean standard error of three experiments (given in parentheses; nd = not determined).
[b]Commercially available, obtained from RBI, TFA salt.
[c]HCl salt.

On the other hand, it is possible that the substitution of Pro with β-(*R*)-Pro gives a tetrapeptide with a more appropriate conformation to bind to μ-opioid receptors than endomorphin-1. This hypothesis is in agreement with the observation that Tyr, Trp, and Phe seem to be important to determine hydrogen bonds and hydrophobic interactions with the receptor, while proline seems to be a spacer that induces the other residues to assume the proper spatial orientation for the ligand–receptor complex formation.[5a,8,20] Although it is possible that endomorphin-1 (**13**) and Tyr-β-(*R*)-Pro-Trp-PheNH_2 (**19**) interact with the receptor in distinct ways, it is also possible that they fit the same site, owing to the tetrapeptide chain flexibility. Moreover, it has been pointed out that a certain degree of flexibility should also be considered for the receptor conformation, allowing molecules with different shapes to bind to the same part of a receptor ('induced fit').[21]

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